

Evidence of an Infectious Virus Reservoir in Suppressed Chronic Hepatitis B Patients

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Abstract

Current approved therapies for Hepatitis B Virus (HBV) effectively control viral replication but are rarely curative. Patients on suppressive therapy still harbor cccDNA and cessation of therapy leads to rapid viral rebound 1-3. Even after long-term therapy of nucleos(t)ide analog (NA) treatment, the majority of chronic hepatitis B (CHB) patients have detectable but not quantifiable HBV DNA ⁴. While these data suggest viral suppression is not absolute with current NA therapies, it has not been determined whether the detectable HBV DNA represents infectious virus. Here, we show that serum from patients on suppressive therapy with viral loads below the limit of quantitation still contain infectious circulating virus. We used a chimeric mouse model with a humanized liver to demonstrate HBV-infected human serum can establish infection similar to that in humans. We determined the limit of detection of the model using diluted patient serum, then measured the infectivity of serum from patients suppressed on therapy. Our results support the hypothesis that residual virus can serve as a reservoir to maintain a chronic infection in patients on therapy. These results highlight the urgent need for orthogonal therapies to eliminate low levels of circulating infectious virus which may minimize ongoing chromosomal damage to the liver and potentially lead to a cure.

Introduction

Hepatitis B is a global epidemic and a major cause of liver disease, including cirrhosis and liver cancer. The WHO estimates approximately 257 million people suffer from CHB worldwide and viral hepatitis accounted for 1.34 million deaths in 2015 (66% due to HBV) ⁵. Widespread vaccination programs have been implemented to control the global prevalence rate of HBV. However, the vaccine does not currently prevent all new infections and is not helpful to patients that already have chronic infection ^{6,7}. Current direct-acting antiviral treatments for CHB target the HBV polymerase. With time, these drugs lead to a suppression of HBV replication in most patients ⁸. Although viral suppression can be achieved in the majority of patients on long-term NA therapy, treatment rarely results in functional cure (e.g HBsAg seroconversion) and patients continue to be at-risk for hepatocellular carcinoma (HCC) ⁹⁻¹². We previously reported that after 240 weeks of TDF or TDF/FTC treatment the majority of CHB patients had detectable (i.e., PCR positive) but not quantifiable (DNQ) HBV DNA (LLOQ \leq 29 IU/mL) ¹³. Moreover, HIV-HBV co-infected patients on long-term TDF therapy still have very low but detectable levels of intrahepatic cccDNA ¹⁴. Taken together, these data suggest that viral suppression is not absolute with the current NA therapies, whether DNQ serum HBV DNA contains infectious virus is unknown. To determine if serum from patients with detectable but not quantifiable HBV DNA contains infectious virus capable of infecting new hepatocytes, we utilized the uPA/SCID chimeric mouse model. Using this mouse model, we assessed the infectivity of serum from patients prior to and after long-term NA therapy.

Results And Discussion

To determine if human serum containing HBV was capable of infection in a mouse model of hepatitis B virus infection, patient isolates from Gilead study GS-US-203-0101 were evaluated in a uPA/SCID mouse model of HBV. SCID mice harboring the urokinase plasminogen activator gene and lacking an intact mouse liver are xenotransplanted with human hepatocytes (**Figure 1**). Mice with reconstituted human livers can be infected with HBV and although the mice lack a functional complete immune system, the disease course is similar to CHB in humans ^{15,16}. In this model, serum HBV DNA, HBsAg and HBeAg levels reach levels similar to those seen in CHB patients. Ultimately, the mice are unable to resolve the infection without intervention ¹⁷. To determine if sera from patients could infect uPA/SCID mice, we identified a set of patients who had been infected with HBV genotype B and C and had high viral loads at baseline (mean viral load of 1.5×10^9 copies/mL) (Figure 2, Supplementary Table 1). Baseline sera from each patient were inoculated by tail vein injection into uPA/SCID mice (n=2 mice/patient serum sample) and monitored for the presence of infection. Mouse sera were analyzed biweekly for HBV DNA by quantitative PCR and HBsAg/HBeAg by ELISA for 112 days. Maximal viremia and antigen production were achieved at between 40 - 60 days post-infection (average 5.5×10^8 copies/mL HBV DNA, 4.5×10^4 IU/mL HBsAg, 3.3×10^3 cut-off index HBeAg) and continued to increase until mice were sacrificed at day 112 post-infection (**Figure 3**). Therefore, baseline patient sera with high viral titers were able to establish infections in uPA/SCID mice.

To determine the lower level of sensitivity of the uPA/SCID mouse model, one patient from each genotype (B or C) was selected for further analysis. Baseline patient sera were serially diluted such that individual mice received 100 μ L of diluted sera containing 100 (n=4), 10 (n=7), 5 (n=3) or 1 (n=3) genome equivalents (GE). Mice were followed for signs of infection for 112 days

by monitoring mouse sera biweekly for HBV DNA by quantitative PCR and HBsAg/HBeAg by ELISA. Mice became viremic when inoculated with human sera containing 100 (4/4, 100%), 10 (6/7, 85%), and 5 (2/3, 67%) GE (**Figure 4A – C**). Mice inoculated with sera containing 1 GE failed to become viremic after 112 days of observation (0/3, 0%) for either genotype tested. Mice inoculated with more virus became viremic faster, with the median time to viremia was 64.5, 94.5, and 96 days for 100 GE, 10 GE, and 5 GE, respectively. As no mice became viremic after inoculated with 1 GE, we can deduce that > 1 and < 5 GE is sufficient for establishing infection in the uPA/SCID mouse model.

Chronic NA therapy is rarely curative as it is hypothesized viral suppression is not complete. Despite reaching clinical suppression (DNQ = <29 IU/mL), viral nucleic acids are still detectable in patient serum. Thus, it is likely there are still low levels of virus being produced, maintaining a pool of infected hepatocytes, even on NA therapy. To confirm if patients suppressed on NA therapy still have low levels of circulating virus, we infected mice with patient serum with viral DNA below the limit of quantitation (**Table 2**). Patients selected for this analysis were infected with either genotype B or C isolates that had been treated with TDF alone or TDF/FTC for 208 weeks (**Figure 2, Supplementary Table 1**). All patients were positive for HBeAg and HBsAg at week 208. The nine patients all achieved viral suppression (≤ 29 IU/mL) at a median of 72 weeks and were classified as having DNQ levels of HBV DNA. Mice (n=14) were inoculated with serum from week 48 and week 96 time points on suppression from 7 different patients. Mouse sera were analyzed biweekly for HBV DNA by quantitative PCR and HBsAg/HBeAg by ELISA. While select mice inoculated with sera from patients near the LLOQ became viremic, none of the mice inoculated with sera below the LLOQ became viremic after 168 days (**Figure 6**).

Given that each 100 μ L inoculation of sera from a patient whose DNA level is below LLOQ (29 IU/mL or 165 copies/mL) may contain less than or equal to 16.5 copies and that every inoculation is subject to the jackpot effect, a separate group of uPA/SCID mice were tested with serial inoculations. Mice were inoculated five times over a two-week period with sera from patients taken 12–32 weeks on NA treatment with HBV DNA ≤ 29 IU/mL (**Supplementary Table 1**). In contrast to the mice receiving single inoculations, mice that received multiple inoculations of sera from patients on NA therapy with DNQ HBV DNA resulted in viremia for 9/31 (29%) samples at a median of 66 days post-infection (**Figure 7A – C**).

NAs have been shown to be safe and effective in improving inflammation and fibrosis, preventing liver failure, reducing the rate of HCC, and improving overall quality of life for patients with HBV. Even after years of NA treatment, the majority of CHB patients still have detectable but not quantifiable HBV DNA and cessation of NA therapy often leads to rapid viral rebound, suggesting the existence of a viral reservoir⁴. Here, we show that sera from patients on long-term NA therapy are capable of infecting naïve hepatocytes in the uPA/SCID mouse model. At the time of this study, only tissue-culture derived laboratory strains or serum from chimpanzees had been used to infect uPA/SCID mice¹⁷⁻¹⁹. Serum from multiple patients and various timepoints below the LLOQ was able to give rise to viremia in mice. In some cases, serum from patients suppressed on therapy for as long 48 weeks (median 34 weeks, range 24 – 48 weeks) was able to cause viremia in mice, indicating that those patients on therapy still contained infectious virus. This is the first demonstration that NA therapy does not fully suppress viral replication and the residual viral DNA likely represents infectious virus.

Consistent with this data, patients who are virally suppressed on NA therapy maintain high-to-detectable levels of serum HBsAg and HBV RNA, and low-to-detectable levels of intrahepatic cccDNA, suggesting that intrahepatic cccDNA is still transcriptionally active. The low-level viremia in NA-treated patients may serve as a viral reservoir leading to the *de novo* formation of cccDNA and maintenance of chronic HBV infection in the liver. The continued low-level viral replication would explain the viral rebound that is observed upon discontinuation of NA therapy. Moreover, the residual viremia may also explain the continued risk, although low, for the development of long-term HBV-related complications, such as HCC, in virally suppressed patients. Studies have demonstrated that patients on NA therapy that achieve functional cure, defined as HBsAg loss, have further reduction in HCC risk compared to those that remain virally suppressed^{20,21}. However, HBsAg loss is uncommon in NA-treated patients^{22,23}. These results highlight the need for orthogonal approaches (i.e., capsid modulators, RNAi, or immune modulation) in addition to NA therapy to achieve HBV functional cure.

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Tables

Supplementary Table 1.

Patient Number	1	2	3	4	5	6	7	8	9
Genotype	B	B	C	C	B	C	B	C	B
Baseline Viral Load (IU/mL)	1.77E+08	1.27E+08	7.38E+08	1.70E+08	3.17E+08	4.73E+08	3.55E+08	5.10E+08	9.45E+07
Treatment	TDF	FTC-TDF	TDF	FTC-TDF	TDF	TDF	FTC-TDF	FTC-TDF	FTC-TDF
Week 4	104000	269000	727000	573000	145000	408000	13800000	617000	185000
Week 8	12600	15400	279000	80100	45300	120000	2690000	169000	47700
Week 16	2440	447	55800	6750	8950	29700	570000	16300	8120
Week 24	164	86	5480	435	1870	1540	102000	1360	1060
Week 32	105	100	1630	131	370	475	18200	211	169
Week 40	193	68	654	99	198	105	4850	138	84
Week 48	254	68	1230	38	107	154	1340	91	29
Week 60	133	29	188	29	49	52	48	81	29
Week 72	34	29	420	29	29	68	29	61	29
Week 84	31	29	214	29	42	191	29	31	29
Week 96	33	29	101	29	107	73	29	29	29
Week 112	37	29	173	29	120	134	29	29	29
Week 128	29	29	117	29	29	81	29	29	29
Week 144	29	29	134	29	29	39	29	29	29
Week 160	29	29	391	29	29	55	29	29	29
Week 176	29	29	436	29	29	29	29	29	29
Week 192	427	29	131	29	29	29	29	29	29
Week 208	29	29	46	29	29	29	29	29	29

Methods

Patient Serum Selection

Nine patients who were infected with HBV genotype B and C viruses were selected from a phase 3 clinical study (GS-US-203-0101) that evaluated TDF alone or in combination with FTC for the treatment of CHB¹⁻⁴. None of the nine patients achieved HBsAg seroconversion. Samples with HBV DNA levels below 169 copies/mL (determined by the COBAS TaqMan assay) were defined as HBV DNA target detected (TD). Of the nine patients selected, only one patient did not become suppressed on treatment. All patient samples were sent to PhoenixBio by Covance biological repository. Unless otherwise specified, inocula were thawed once, aliquoted once to limit freeze thaw cycles, and maintained at -80°C. Patient serum was kept frozen until the day of the study and thawed at 37°C. All mice received intravenous injections (IV) of inocula via the tail vein using disposable 1.0 mL syringes with permanently attached needles. For dilution studies, patient serum was diluted in serum of cDNA-uPA^{wild/+}/SCID mice that had not been transplanted with human hepatocytes to achieve the desired target concentration. The desired target concentration was validated by quantitative real-time PCR with at least 5 technical replicates.

Study oversight

All patients provided informed consent. The study was approved by the Institutional Review Board at both participating sites and was conducted in compliance with the Declaration of Helsinki, Good Clinical Practice guidelines, and local regulatory requirements. The study was designed and conducted by the sponsor in collaboration with the principal investigators. The sponsor collected the data and monitored the study conduct. The investigators, participating institutions, and sponsor agreed to maintain confidentiality of the data. All authors had access to the study data and reviewed and approved the final manuscript.

In vivo mouse studies

Male uPA/SCID mice between 12-18 weeks of age at study day 0 with humanized liver (cDNA-uPA^{wild/+}/SCID [cDNA-uPA^{wild/+}: B6;129SvEv-Plau, SCID:C.B-17/Icr-scid /scid Jcl) were purchased from PhoenixBio, Co., Ltd., (Japan) and produced as described⁵. Frozen human hepatocytes (donor BD195, Corning Incorporated, Tewksbury, MA, USA) were thawed and transplanted into 2- to 4-week-old uPA/SCID mice by splenic injection. Mice were selected for studies if they contained hepatocytes with an estimated replacement index greater than 70% based on the blood concentration of human albumin (>8.5mg/mL) at one week prior to study onset. General health observations including weight were monitored weekly. Within the different groups, serum HBV DNA sampling frequency ranged between 30 minutes and 9 days with sampling in the different groups beginning as soon as 1 minute post-infection and continuing through 63 days after inoculation. All animal protocols described in this study were performed in accord with the Guide for the Care and Use of Laboratory Animals and approved by the Animal Welfare Committee of Phoenix Bio Co., Ltd. All mice were housed individually and maintained in accordance with the Animal Ethics Committee of PhoenixBio (resolution #1856, 1916, 2104, 1943, 2102, and 2103). In total, all studies described herein included 94 uPA/SCID mice with humanized livers divided into 4 experimental groups.

Blood was collected from all animals under isoflurane (ISOFLURANE Inhalation Solution [Pfizer], Mylan, Osaka, Japan) anesthesia via the retro-orbital plexus/sinus using Intramedic™ Polyethylene Tubing (Becton Dickinson and Compound, NJ, USA) at each time point. A maximum of 40 µL was obtained at each timepoint. For blood collection at terminal sacrifice, a minimum of 400 µL was collected by cardiac puncture and exsanguination. Of the blood collected, 2 uL was reserved for human albumin measurement. Remaining blood samples were left at room temperature for at least 5 minutes to coagulate and then centrifuged at 13200 x g at 4°C for 3 minutes to obtain serum.

Quantification of Human Serum Albumin

Blood obtained was diluted in saline and the clinical chemistry analyzer (BioMajesty™ Series JCA-BM6050, JEOL Ltd., Tokyo, Japan) was used to measure the albumin concentration using latex agglutination immunonephelometry test (LX Reagent “Eiken” Alb II; Eiken Chemical Co., Ltd., Tokyo, Japan). Levels of human serum albumin were measured as soon as 1 minute after inoculation and then at weekly intervals.

Extraction and Quantification of Serum HBV DNA

DNA was extracted from 10 µL (5 uL x 2) of serum by the Smitest Ex-R&D Nucleic Acid Extraction Kit (Medical & Biological Laboratories Co, Ltd, Nagoya, Japan) and was dissolved in 20 µL of nuclease-free water (Life Technologies Japan Ltd., Tokyo, Japan). HBV DNA copy numbers were determined by quantitative real-time PCR (qRT-PCR) as reported previously. HBV DNA extracted from the serum of an HBV-infected uPA/SCID mouse was used as the DNA standard. The copy numbers in the standard were determined in reference to a synthetic HBV DNA template. The range of standards used was 4×10^4 to 2×10^9 copies/mL. The lower limit of quantification is 4×10^4 copies/mL. The lower limit of detection is 8×10^2 copies/mL. Real-time quantitative PCR was performed using the TaqMan Fast Advanced Master Mix (applied Biosystems, Thermo Fisher Scientific Inc.) and ABI Prism 7500 sequence detector system (Applied Biosystems) using a forward probe (HBV target location 166-186: 5' -

CACATCAGGATTCCTAGGACC - 3'), reverse probe (HBV target location 344-325: 5' – AGGTTGGTGAGTGATTGGAG – 3') and TaqMan probe (HBV target location 242 – 267: 5' - 6-FAM-CAGAGTCTAGACTCGTGGTGGACTTC-TAMRA – 3'. The initial activation of uracil-N-glycosylase at 50°C for 2 minutes was followed by the polymerase activation at 95°C for 30 seconds. Subsequent PCR amplification consisted of 53 cycles of denaturation at 95°C for 3 seconds and annealing and extension at 60°C for 32 seconds per cycle. The Ct values generated were used to determine HBV DNA copies/mL based on the DNA standard.

Serum HBV Antigen Quantification

Serum was sampled biweekly for HBsAg and HBeAg quantification. Serum samples were diluted 120-fold before analysis and measured in duplicate. Serum HBsAg concentration was determined by SRL, Inc. (Tokyo, Japan) based on Chemiluminescent Enzyme Immuno Assay (CLEIA) developed by Fujirebio (LUMIPULSE HBsAg-HQ, LUMIPULSE® Presto II). The detection range of the assay was between 5×10^{-3} and 9.99×10^7 IU/mL. After dilution, the detection range was adjusted to be between 6×10^{-1} and 1.2×10^{10} IU/mL. Serum HBeAg concentration was determined by SRL, Inc. based on Chemiluminescent Enzyme Immuno Assay (CLEIA) developed by Fujirebio (LUMIPULSE HBeAg, LUMIPULSE® Presto®). The detection range of this assay was between 1×10^{-1} and 1.6×10^3 C.O.I. After dilution, the detection range was adjusted to be between 1.2×10^1 and 1.9×10^5 C.O.I. Cut-off index (COI) is based on the ratio of assay signal to cut-off signal (also abbreviated s/co).

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Declarations

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AUTHOR CONTRIBUTIONS

D.L.B. designed experiments and managed the project. All authors designed the experiments and interpreted the results. H.L.Y.C. managed the clinical trial from which patient serum samples were sourced. D.L.B. wrote the paper with input from all authors.

COMPETING INTEREST DECLARATION

D.L.B., S.L., J.Y., W.E.D., and B.F. are current or previous employees of Gilead Sciences (except H.L.Y.C.) and have received a salary and stock ownership as compensation for their employment. The study was funded in full by Gilead Sciences, Inc. HLYC is an advisor of AbbVie, Aligos, Arbutus, Hepions, Gilead Sciences, Janssen, Merck, Glaxo-Smith-Kline, Roche, Vaccitech, VenatoRx, Vir Biotechnology, and a speaker for Gilead Sciences, Mylan and Roche.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ADDITIONAL INFORMATION

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Figures

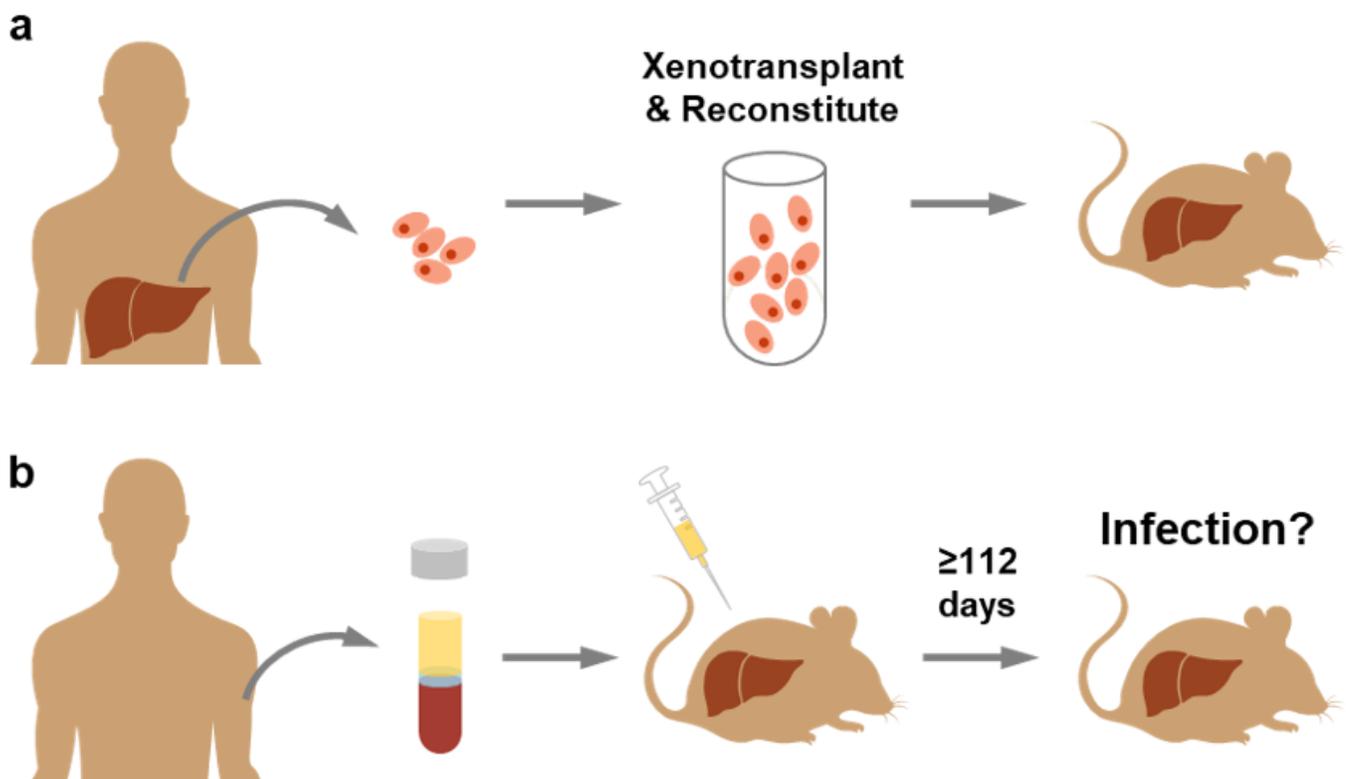


Figure 1

Experimental overview of testing patient serum in the human liver chimeric mouse model. a, Cryopreserved human hepatocytes were transferred into 2-4 week old male uPA/SCID mice by splenic injection and validated based on a serum albumin levels for > 70% replacement index. b, uPA-SCID mice were inoculated with sera from patients on NUC therapy and monitored for evidence of viremia for ≥ 112 days.

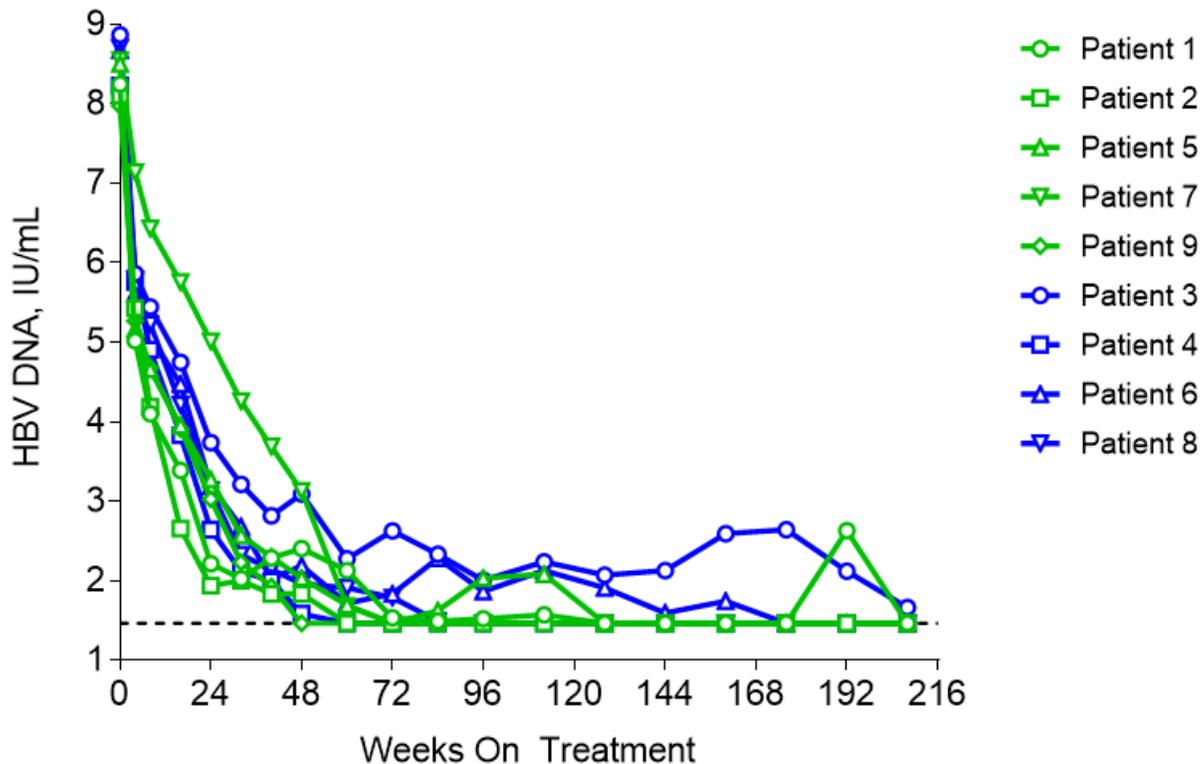


Figure 2

Viral kinetics of 9 patients on antiviral treatment. HBV DNA was measured by Roche TaqMan COBAS at the indicated timepoints and reported as international units per milliliter (IU/mL). Patients infected with genotype B virus are colored green and patients infected with genotype C virus are colored blue. The dashed line represents the lower limit of quantitation (LLOQ) of the assay, 29 IU/mL.

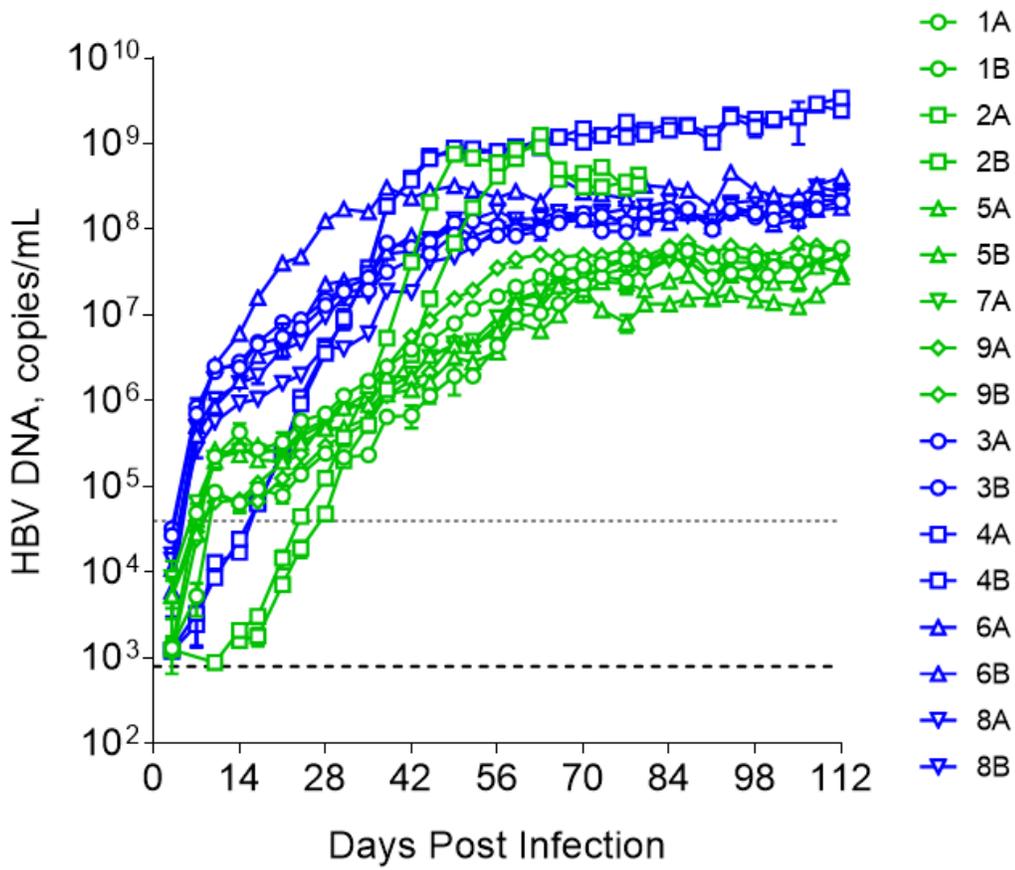


Figure 3

Viral kinetics of uPA/SCID mice infected with patient-derived virus. uPA/SCID mice (n=2 per isolate reflected as A and B) were infected with 0.1 mL of baseline serum samples from patients infected with HBV genotypes B (green lines) and C (blue lines) prior to treatment onset. HBV DNA was measured by qRT-PCR and reported as copies/mL. The small dashed line represents the LLOQ (4×10^4 copies/mL) and the large dashed line represents the LLOD (8×10^2 copies/mL).

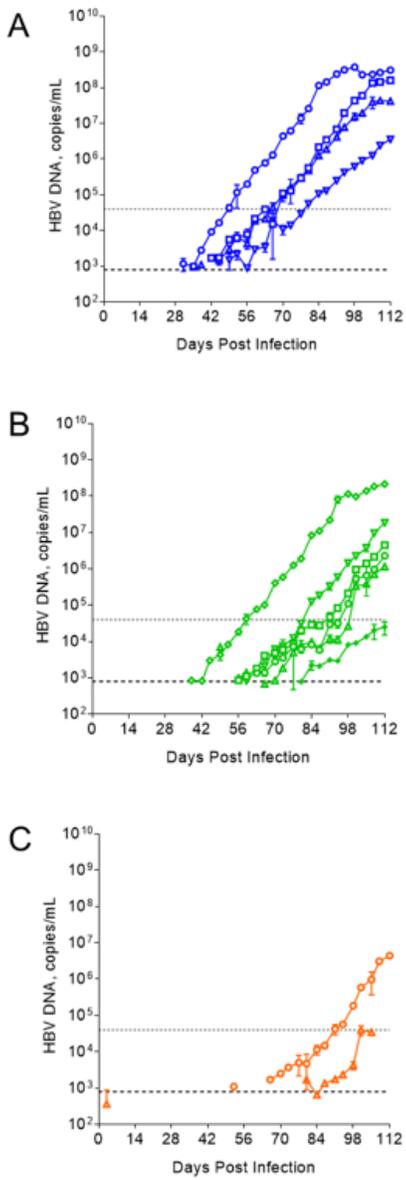


Figure 4

Establishment of Lower Limit of Detection in uPA/SCID mice. uPA/SCID mice were infected with 0.1 mL of sera from patients infected with HBV containing 100 GE (A, n=4), 10 GE (B, n=7), and 5 GE (C, n=3). HBV DNA was measured by qRT-PCR and reported as copies/mL. The small dashed line represents the LLOQ (4×10^4 copies/mL) and the large dashed line represents the LLOD (8×10^2 copies/mL).

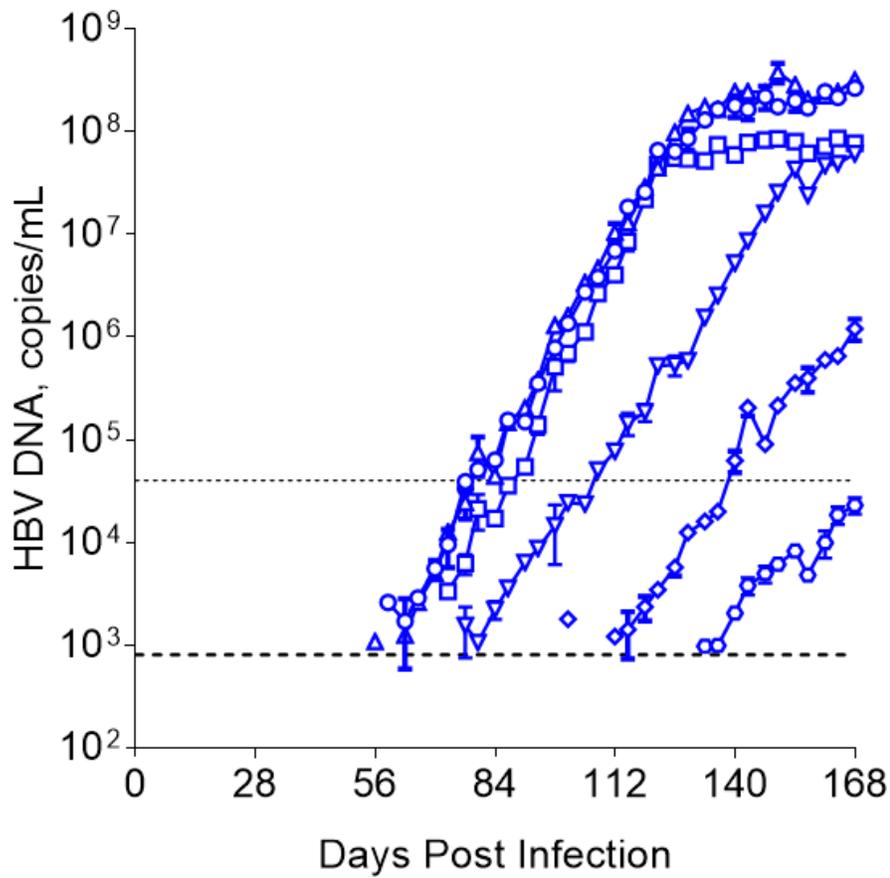


Figure 5

Patient Serum Near the Limit of Detection are Infectious. uPA/SCID mice were infected with 0.1 mL of sera from patients infected with HBV where the HBV DNA levels were at or below the LLOQ. HBV DNA was measured by qRT-PCR and reported as copies/mL. The small dashed line represents the LLOQ (4×10^4 copies/mL) and the large dashed line represents the LLOD (8×10^2 copies/mL).

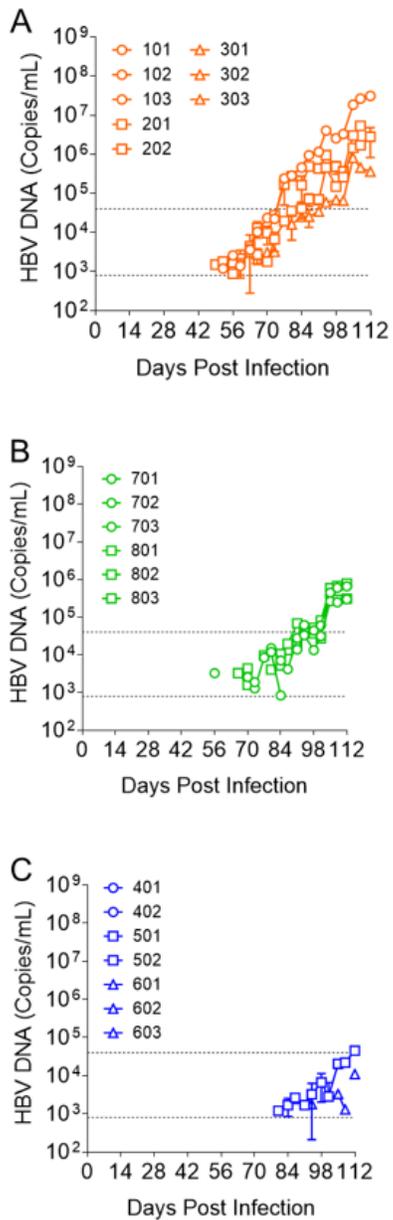


Figure 6

Patient Serum Below the Limit of Detection are Infectious. uPA/SCID mice were infected with a total of 1.0 mL of sera (0.2 mL x 5) from patients infected with HBV where the HBV DNA levels were below the LLOQ. HBV DNA was measured by qRT-PCR and reported as copies/mL. The small dashed line represents the LLOQ (4×10^4 copies/mL) and the large dashed line represents the LLOD (8×10^2 copies/mL).